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Behaviour of pill millipedes can be affected by external marking

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Abstract

External or internal marking techniques are often used in various studies of invertebrates to distinguish individuals. Although the potential influence of marking agents on survival is often evaluated, a possible effect on behaviour is usually neglected. We evaluated the influence of two external marking agents (nail polish and bee marker) on behaviour of the pill millipedes, *Glomeris tetrasticha*, in laboratory. Behaviour was examined from two points of view: (1) specific expressions of behaviour (feeding, exploring, resting and hiding) through 24 hours and (2) activity through 24 hours. The nine-day experiment compared behaviour between groups marked with nail polish or bee marker and the control group. Although there was no observed influence of marking on survival, there was an evident influence on the frequency of feeding, resting and hiding. An effect on frequency of exploring was significant in the marker-marked group only. Marked individuals of *G. tetrasticha* also differed from the control group in overall activity. They were less active overall and preferred resting and hiding. *G. tetrasticha* were found to be quite active during almost the whole day in the laboratory, with maximum feeding behaviour in the early morning.

Keywords

external marking, influence on behaviour, diurnal activity, *Glomeris tetrasticha*

Introduction

Zoologists need to distinguish individuals of model species occasionally. It is often important in ecological investigations (e.g. capture-mark-recapture studies of population size or migration studies) as well as in ethological studies (e.g. studies of home range,

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shelter fidelity or social hierarchy). Although it is possible to recognise individuals of some species by their natural characteristics, e.g. long-lived mammals, this may not be possible for short-lived invertebrates. Special methods therefore been developed for invertebrate marking studies. Internal marking methods are based on feeding individuals with coloured food (Evans and Gleeson 1998) or using subcuticular injection of a coloured medium (Chapin 2011). These methods are suitable mainly for unpigmented animals (e.g. termites, tiny spiders, some geophilomorph centipedes). Other internal marking methods rely on radioactive or stable isotopes, but isotopes have mainly been used in population studies (Southwood and Henderson 2000), e.g. Paris (1965) used this method in a study of pill woodlouse dispersal. External marking methods are more frequently used in studies of invertebrates, especially of the final developmental (non-moulting) stage of insects. Beside mutilation (e.g. deformations of beetle elytra by rasp or laser) and tagging (labels with code on locusts, molluscs etc.), painting is one of the most popular methods of external marking. Painting of invertebrates has been used in studies of life history (Lawlor 1976, Madhavan and Shribbs 1981), shelter fidelity (Brereton 1957, den Boer 1961) and vagility (Paris and Pitelka 1962). Several experiments were conducted to test the durability of various external marking agents on the millipede *Ommatoiulus moreletii* (Lucas, 1860), an invasive exotic species in Australia (Petit et al. 2003, Penny et al. 2005, Petit and Gibbs 2005, Gordon et al. 2007). All tested agents had low durability and were lost from millipedes during burrowing in a quite short time.

Marking might affect survival (probability of predation or infection, intoxication) as well as behaviour of marked individuals. Potential effects of marking on survival of marked animals are often evaluated but effects on behavior are overlooked. We therefore investigated the influence of two external markers (nail polish and bee marker) on behaviour of the pill millipede, *Glomeris tetrasticha* Brandt, 1833. Our study was also aimed at evaluating possible effects of marking on survival.

Materials and methods

Biological material and marking process

Pill millipedes *Glomeris tetrasticha* were hand-collected in the floodplain forests in Litovelské Pomoraví, Protected Landscape Area near Olomouc City (Czech Republic). Collected animals were kept in plastic boxes under laboratory conditions (room temperature, 100% humidity, natural summer photoperiod, food ad libitum). Three groups, each containing 40 similar-sized individuals, were chosen for the experiment. The first two were marked while the third group was left unmarked and served as a control.

Two external marking agents selected for the experiment were nail polish (60 seconds RIMMEL London™) and bee marker (Uni Paint Marker™). The fast-drying nail polish was used to reduce the probability of bonding tergites or sticking of an individual to the substrate. Animals were held gently between two fingers, marked quickly with a small dot of marking agent on the joined second and third tergite and placed

back into the box. The control group was also manipulated, i.e. handled the same way but not marked.

Experimental design

The experiment was performed during August 2009. Individuals from polish-marked, marker-marked and control groups were placed in sets of four into 20×20×10 cm boxes with a 0.5 cm layer of plaster. One box with four randomly chosen individuals from one group was considered as one sample. Each box was divided into thirds: the first third contained 40 g of fine soil, the second third contained three shelters made from dark but see-through red plastic and the last third contained three pieces of potatoes as food. After sunset a red-coated flashlight was used for illumination to minimize the disturbance of individuals. There were 10 repetitions in each treatment, i.e. 30 boxes altogether. After the marking process, individuals were left to acclimatize in the experimental box for two days. Observations were performed for 24 hours on the 3rd, 6th and 9th day. Individual behaviour was recorded hourly and categorised as: hiding (inactivity in soil or in shelter), resting (inactivity on surface), exploring (walking), monitoring (standing with moving antennae), cleaning (cleaning of antennae or legs), interacting (contact with another individual) or feeding (feeding on potatoes, excrement or soil, drinking or defecation).

Statistical analysis

Generalized additive models (GAMs) in R software (http://www.r-project.org/) were used to analyse differences in frequencies of behavioural categories between marked and control groups. Activity in each 24 hours was also analyzed with GAMs, but additional analyses and visualizations were done using Oriana software (http://www.kovcomp.com/oriana/oribroc.html).

Results

In total, 8640 observations of behaviour pattern were recorded, but cleaning behaviour was never observed and interacting or monitoring behaviour was too rare for evaluation (16 and 138 observations respectively). An effect on behaviour was observed in most cases at first sight: animals looked apathetic and unhealthy. Not only were there evident differences between marked and control groups, but changes in overall behaviour pattern during the experiment were also observed. Gradual increase in feeding and decrease in exploring were the most evident examples.

Significant temporal pattern was found in feeding in all observation days (Tab. 1): millipedes fed regularly between ca 2300 and 0600 hrs. Individuals from the control group fed significantly more often than those from marked groups on all three obser-

Table 1. Analyses of effect of time on frequency of behaviour categories and total activity of *G. tetrasticha* in GAMs.

	FEEDING		EXPLORING		RESTING		HIDING		ACTIVITY	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
3rd day	48.19	< 0.001	28.93	< 0.001	1.32	0.251	68.13	< 0.001	44.92	< 0.001
6th day	39.01	< 0.001	8.16	0.189	0.29	0.588	74.78	< 0.001	42.29	< 0.001
9th day	50.85	< 0.001	21.56	0.007	23.54	0.003	41.25	< 0.001	47.26	< 0.001

Table 2. Analyses of effect of marking on frequency of behaviour categories and total activity of *G. tetrasticha* in GAMs.

		FEEDING		EXPLORING		RESTING		HIDING		ACTIVITY	
		z value	*p*	*z value*	*p*	*z value*	*p*	*z value*	*p*	*z value*	*p*
3rd day	CONTROL (intercept)	-19.10	< 0.001	-18.90	< 0.001	3.42	< 0.001	-20.30	< 0.001	-10.24	< 0.001
	MARKER (x CONTROL)	-7.84	< 0.001	-6.26	< 0.001	7.84	< 0.001	0.49	0.622	-9.87	< 0.001
	POLISH (x CONTROL)	-7.62	< 0.001	-1.89	0.059	3.53	< 0.001	1.90	0.057	-5.69	< 0.001
6th day	CONTROL (intercept)	-8.70	< 0.001	-19.80	< 0.001	-2.58	0.010	-19.70	< 0.001	-4.86	< 0.001
	MARKER (x CONTROL)	-11.60	< 0.001	-1.39	0.166	11.53	< 0.001	-1.41	0.158	-11.44	< 0.001
	POLISH (x CONTROL)	-11.30	< 0.001	1.19	0.235	9.75	< 0.001	-1.95	0.052	-9.11	< 0.001
9th day	CONTROL (intercept)	-11.40	< 0.001	-19.80	< 0.001	-0.46	0.643	-18.90	< 0.001	-7.74	< 0.001
	MARKER (x CONTROL)	-8.22	< 0.001	-2.68	0.007	9.47	< 0.001	-2.71	0.007	-8.40	< 0.001
	POLISH (x CONTROL)	-6.85	< 0.001	0.08	0.940	4.55	< 0.001	1.341	0.180	-5.96	< 0.001

vation days (Figs 1a–c, Tab. 2). Exploring by millipedes in spite of marking showed significant time-pattern in the 3rd and 9th day (Tab. 1): millipedes were exploring boxes in late evening (ca 2200 hrs) and in the morning (ca 0900 hrs). Although there were no significant differences in the frequency of exploring between millipedes from the control group and millipedes from the polish-marked group, millipedes marked by bee-marker were exploring significantly less on the 3rd and 9th observation days (Figs 1d–f, Tab. 2). Resting was recorded at any time on the 3rd and 6th observation days; millipedes showed significant temporal pattern of resting only during the last day of the experiment (Tab. 1). On this day, millipedes rested mainly during the afternoon with a peak at ca 1500 hrs. Regardless of time pattern, millipedes from both marked groups rested significantly more during the whole of the experiment (Figs 1g–i, Tab. 2). Unlike resting, hiding showed a significant temporal pattern over the three observation days (Tab. 1). Millipedes were hidden especially during afternoon and evening (between ca 1500 and 1800 hrs). Marked millipedes did not hide more frequently

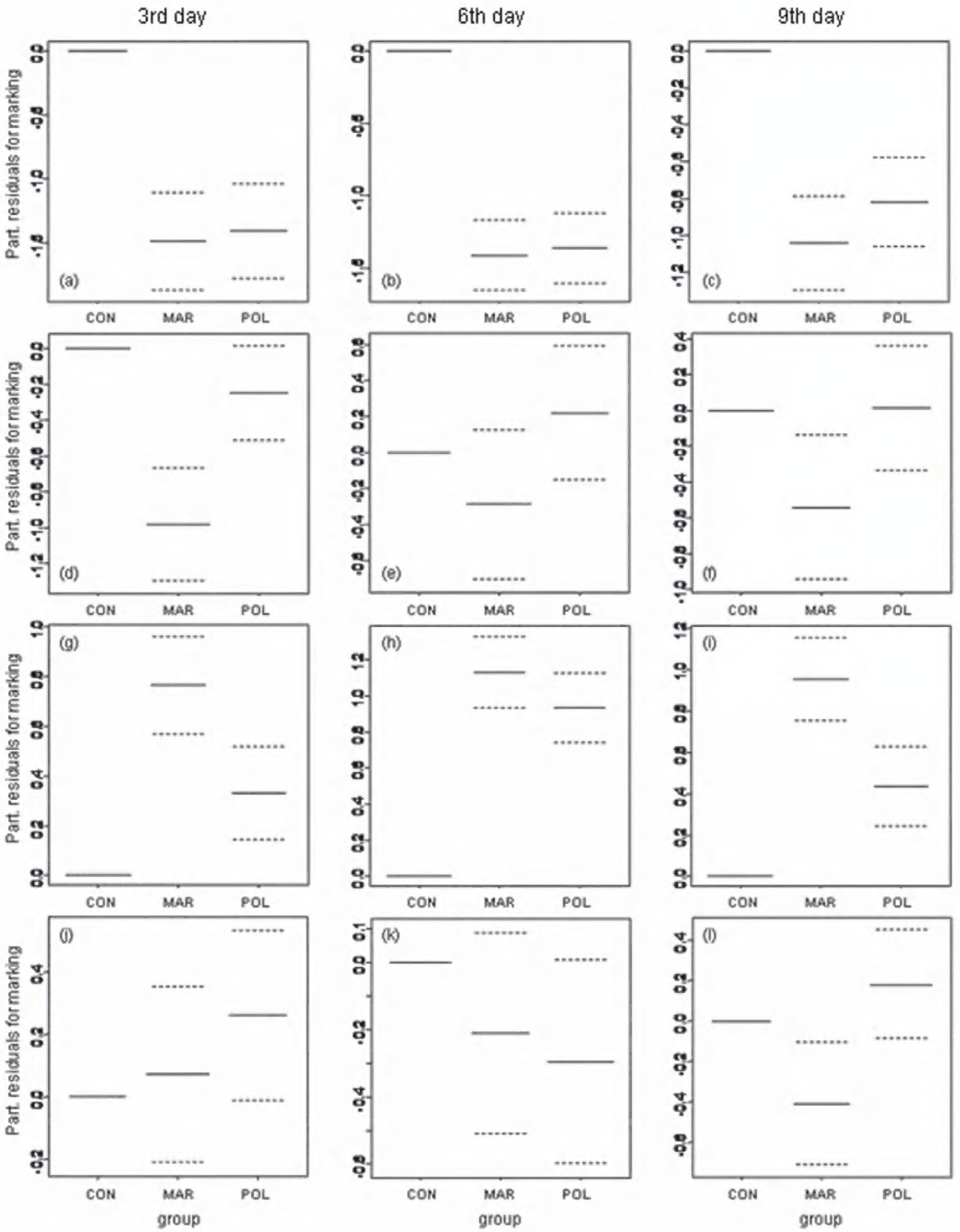

Figure 1. Influence of marking on feeding **a**, **b**, **c** exploring **d**, **e**, **f** resting **g**, **h**, **i** and hiding **j**, **k**, **l** of *G. tetrasticha* on the 3rd, 6th and 9th days analyzed by GAMs, confidence intervals dotted. Legend: CON – control, MAR – marker-marked, POL – polish-marked.

compared to millipedes from the control group except for the bee-marker-marked group on the 9th day (Figs 1j–l, Tab. 2).

In evaluating the distribution of both active categories of behaviour (i.e. feeding and exploring), there are evident differences between activity level of millipedes in the

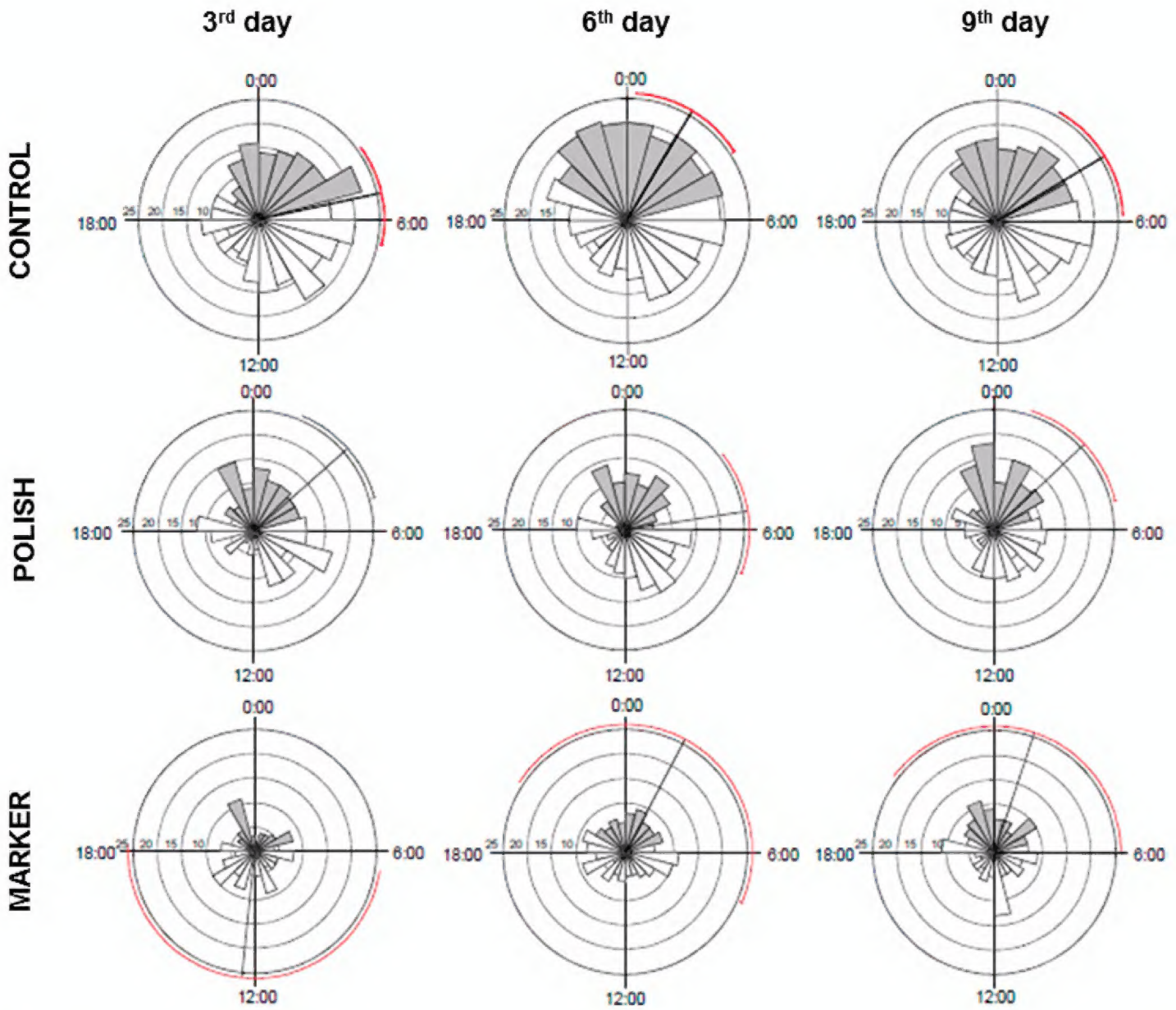

Figure 2. Temporal-pattern of frequency of activity (feeding and/or exploring) of *G. tetrasticha* from all groups on observation days. Scale for triangles shows number of observations, grey triangles mark night-time activity, black line running from the centre of the diagram to the outer edge marks mean time of activity and the arcs extending to either side represent the 95% confidence limits (black arcs are statistically significant).

control group and millipedes in the two marked groups (Fig. 2). Activity of millipedes from both marked groups was significantly lower on all observation days (Tab. 2) but showed a similar significant time-pattern (Tab. 1). Although millipedes were active over the whole day, their activity increased after sunset and stayed high until ca 1000 hrs with mean time of activity during second half of night (i.e. between ca 0100 and 0500 hrs, except marker-marked millipedes in the 3rd observation day; Fig. 2).

We did not find any difference between survival of millipedes in the control group compared to millipedes from the polish-marked group (p=0.675) or millipedes from the marker-marked group (p=1.000).

Regardless of marking, the temporal pattern of a typical pill millipede's daily behaviour can be expressed as a compound activity graph showing the probability of expression of behavioural categories over 24 hours (Fig. 3; we did not employ a curve for resting, as millipedes usually rested occasionally throughout the whole day). Pill millipedes, *G. tetrasticha*, usually feed at night and hide during the day.

Discussion

We evaluated the effect of two external marking agents (nail polish and bee-marker) on the behaviour and mortality of the pill millipede *Glomeris tetrasticha*. Neither agents had any effect on survival of millipedes, but an influence on their behaviour was evident in almost all studied cases. Millipedes of both marked groups were less active, fed less and rested more compared to those from the control group. Millipedes marked by bee-marker also explored less. Similar results were found in a study by Gallepp and Hasler (1975) of marked caddisfly larvae. Marked larvae were hidden in their boxes more than unmarked larvae. Authors attributed their results to marking and manipulation. Nevertheless, we were not completely sure about a possible effect of short gentle manipulation even after nine days. Moreover, we manipulated control and experimental (marked) millipedes identically (see Material and methods).

Pill millipedes were significantly less active due to marking. In our parallel study with the common pill woodlouse *Armadillidium vulgare* (Latreille, 1804), marking also affected behaviour of woodlice significantly, but to a lesser extent (Drahokoupilová and Tuf 2011). Marked woodlice fed less and rested and hid more compared to the control group. Woodlice marked by polish explored less also. The different level of effects on woodlice and millipedes may have an anatomical basis. The thin cuticle of *G. tetrasticha* is very permeable to water (Edney 1951) in comparison not only with the thick cuticle of *A. vulgare*, but also with that of other millipedes (Hopkin and Read 1992). Chemicals in polish and bee-marker might penetrate through the cuticle into the haemolymph of pill millipedes, and lower activity and greater resting could have been a result of some poisoning. The bee marker probably does not affect behaviour of marked bees, because the dot of marking agent is not in contact with cuticle but with hairs only (Sammataro and Avitabile 1978). The possible intoxication by marking agents results in similar behaviour to that reported for parasitized Seychelles giant millipede *Secheleptus seychellarum* (Desjardins, 1834). In dry years, a high proportion of the population of this species is parasitized by larvae of a sarcophagid fly. Affected millipedes were less active and spent more time inactive on the surface outside shelters, resulting in death (Gerlach et al. 2005). From this point of view, rapid activity resulting in removal of painted dots, as observed in O. *moreletii* in experiments done by S. Petit's group (Petit et al. 2003, Penny et al. 2005, Petit and Gibbs 2005, Gordon et al. 2007) looks like a defensive strategy available mainly to the "bulldozer" eco-morphological type (Hopkin and Read 1992).

It is evident that *G. tetrasticha* explore their surroundings at the start of night and in the morning (Fig. 3). Our interpretation is that they look for food at night and for shelter in the morning, and spend all day hidden. We know *G. tetrasticha* is active mainly from 2100 to 0900 hrs with a peak at ca 2300 hrs (Tuf et al. 2006, misidentified as *G. connexa*) in central European woodlands. Activity in field conditions was measured as falling into traps during epigeic moving. The walking category

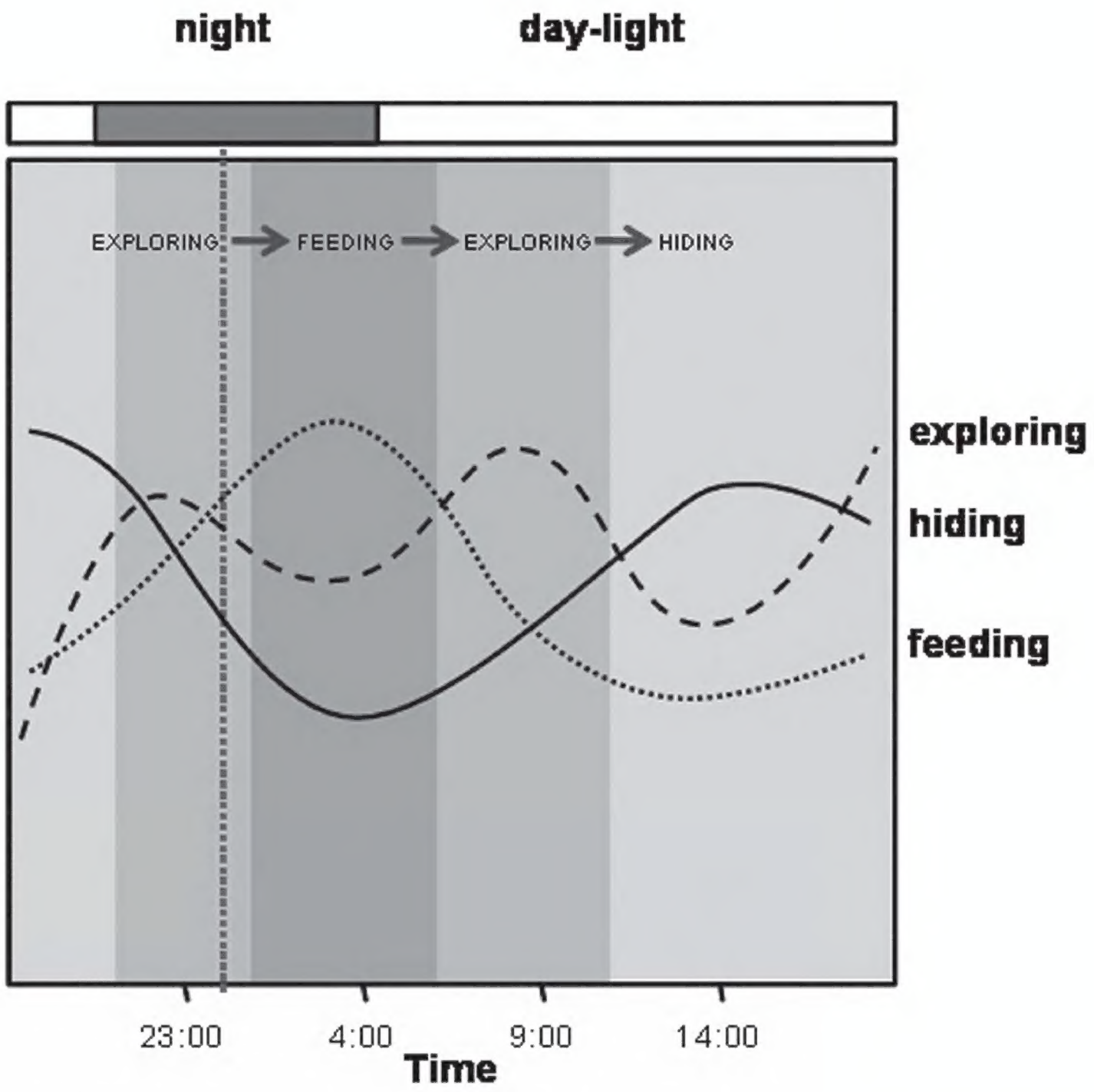

Figure 3. Time-pattern of behaviour of *G. tetrasticha* composed from curves expressing relative frequency of exploring, feeding and hiding.

of behaviour in the laboratory was evaluated as exploring with peaks at 2200 and 0900 hrs. Although exploring behaviour is a part of feeding behaviour, the proportion of time spent during the night in feeding is larger than that spent in looking for another piece of food. That may be a reason, why for the controls higher proportion of feeding was recorded compared to exploring during the night. Exploring during day (cf. Fig. 2) was also noted in the field: "We repeatedly found several specimens of *Glomeris* on paths or similar exposed surfaces in the direct sunlight on hot summer days" (Tuf et al. 2006).

We conclude that millipedes (at least the pill millipede *Glomeris tetrasticha*) should be externally marked neither with nail polish nor with bee-marker. Both marking agents cause lower activity of marked millipedes and their usage (e.g. in capture-mark-recapture studies) can provide biased or false results.

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